

AD-A206 646

TOP SECRET COPY

To: J. Leukocyte Biology

(2)

Contract N00014-87-K-0216

Activated Langerhans Cells release Tumor Necrosis Factor

James W. Larrick MD PhD<sup>1,4</sup>, Vera Morhenn MD<sup>2</sup>

Yawen L. Chiang PhD<sup>3</sup> and Tim Shi MD<sup>3</sup>

STIC  
ELECTE  
MAR 17 1989  
D & D

<sup>1</sup>Psoriasis Research Institute, Palo Alto, CA 94305

<sup>2</sup>Department of Dermatology, Stanford Medical Center, Palo Alto, CA 94305

<sup>3</sup>Department of Immunology, Cetus Corp., Palo Alto, CA 94303

<sup>4</sup>Correspondence: Dr. James W. Larrick, Genelabs Inc.

505 Penobscot Drive

Redwood City, CA. 94063

Running Title: Langerhans cells release TNF

Key words: Langerhans cells, TNF, monokine, cytokine, inflammation

DISTRIBUTION STATEMENT A  
Approved for public release  
Distribution Unlimited

## ABSTRACT

Langerhans cells act as antigen presenting cells in immune reactions in the skin. What other roles they may play in inflammation are less well defined. We have tested whether these cells can produce TNF-alpha, an important mediator of inflammation. Resting Langerhans cells produce less than 0.1 U TNF-alpha/ml. Langerhans cells stimulated with phorbol myristate acetate (PMA) and lipopolysaccharide (LPS) release 4-5 U TNF-alpha/ml. Specificity of the released TNF-alpha in an L929 cytotoxicity assay was confirmed using neutralizing anti-TNF-alpha monoclonal antibodies, and the identity of TNF-alpha was further confirmed by Northern blot hybridization using a TNF-alpha oligomer DNA probe. Activated Langerhans cells may contribute to inflammation in the skin by releasing TNF-alpha, which is known to effect fibroblast growth, endothelial cell activation, and lymphocyte function. (KT)



Accession For	
NTIS	DTIC
DTIC	Unannounced
Justified	
By <i>per NP</i>	
Date	
Availability	
Dist	Availability
<i>A-1</i>	

## INTRODUCTION

Langerhans cells (LC) are mononuclear, dendritic cells located among suprabasal keratinocytes in the epidermis of all mammalian species. Although they represent only 2-6% of all epidermal cells they have a number of important roles in the initiation and maintenance of the immunoinflammatory response (1-2). For example they express class II antigens (3-5), the CD1 (OKT6) antigen (6) and the CD4 antigen (7) and they bear receptors for the Fc portion of IgG and several complement components (8). They appear to act as accessory cells in the induction of the mixed skin cell lymphocyte reaction (5,9).

Mononuclear phagocytes are known to produce and release a large number of biologically active cytokines (10). Two of these, interleukin 1 and tumor necrosis factor (TNF-alpha), are important mediators of the immunoinflammatory response (11). In the skin human keratinocytes are known to produce interleukin 1 alpha and beta, formerly called ETAF (epidermal cell-derived thymocyte activating factor) (12-13). LCs also release an IL-1 activity (14). We have tested whether these cells also produce TNF-alpha. Stimulated but not resting LCs contain mRNA for TNF-alpha and release this inflammatory mediator.

## **METHODS**

### **Preparation of Dispersed Skin Cells**

Single cell suspensions of normal skin were prepared from skin obtained at surgery as described previously (15-16). Briefly, trimmed skin was cut into 1 x 5 cm strips and split-cut with a Castroviejo keratome set at 0.1 mm. The resulting slices were treated for 45 minutes at 37°C with 0.3% trypsin (M.A. Bioproducts, Walkersville, MD.) plus 0.1% EDTA in GNK (0.8% NaCl, 0.04% KCl, 0.1% glucose, 0.084% NaHCO<sub>3</sub>, pH 7.3). Dispersed cells were suspended in Dulbecco's MEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 50 ug/ml gentamicin, and 2 mM L-glutamine (complete medium). Viability as determined by trypan blue exclusion immediately after trypsinization, was 80 percent or better.

### **Ficoll-Hypaque (F/H) Gradient Separation**

Dispersed epidermal cells were resuspended at  $4 \times 10^6$  cells per ml in complete medium. Five ml of this cell suspension was layered on top of 3 ml Ficoll-Paque (Pharmacia, Piscataway, NJ) in a 15 ml tube. The tubes were centrifuged at 400 x g for 30 minutes at ambient temperature. The LC enriched interphase was collected, washed once, and resuspended in complete medium.

## Panning

A 100 x 25 mm Lab Tek petri dish (Scientific Products) was coated with 10 ml of goat anti-mouse immunoglobulin (Zymed Labs Inc. South San Francisco, CA) at 10 µg/ml in 0.05 M Tris buffer, pH 9.5, for 40 min. hour and then washed three times with phosphate buffered saline (PBS) and once with 5% FCS/PBS. The epidermal cells were incubated with OKT6 monoclonal antibody (6) for 20 minutes, washed, resuspended in 5% FCS/PBS, layered on the coated petri dish surface, incubated at 4°C for 40 minutes, swirled, and incubated for another 30 minutes (16). The supernatant containing unattached cells was poured off gently, and the petri dish surface was washed five times with 1% FCS/PBS. The cells in the first three washes were pooled with the supernatant cells, and the cells in the last two washes were discarded. Attached Langerhans cells were scraped off with a rubber policeman in 1% FCS/PBS, centrifuged and resuspended in complete medium.

## Induction of Natural Tumor Necrosis Factor (nTNF) from Langerhans Cells (LC).

Both LC enriched cells and LC depleted cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS). Phorbol myristate acid (PMA) was added at a final concentration of 100 ng/ml. The cells were incubated for 30 minutes at 37°C, collected by centrifugation, and resuspended in medium containing 10 µg of Escherichia coli 0111:B4 bacterial endotoxin (LPS). The cells were incubated at 37°C for 24 hours.

The culture supernatant were harvested and assayed in the TNF bioassay later the same day. Controls were the same cells without any stimulation.

#### **MTT Assay for Measuring Cytotoxic Activity.**

An MTT (1-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; M-2128 Sigma Chem. Co., St Louis, MO) cytotoxicity assay was used to measure inhibition of cell growth and % dead cells. In brief, murine L929 target cells were seeded in microplates, at a concentration of  $4 \times 10^4$  cells/well in 100  $\mu$ l medium. Different dilutions of rTNF-alpha in RPMI-1640 was added to the target cells as standard control. The rTNF-alpha used in this study had a specific activity of  $1 \times 10^7$  U/mg protein in a 24 h bioassay with actinomycin D-treated L929 cells (17). After 18-20 h of incubation at 40°C, 20  $\mu$ l MTT at a concentration of 2.5 mg/ml in phosphate-buffered saline was added and further incubated for 4 h at 37°C. After aspirating all the supernatant from the wells, 100  $\mu$ l H<sub>2</sub>O and 100  $\mu$ l isopropanol with 0.04 N HCl was added to all wells. After dissolving the dark blue formazan crystals, the plates were read on a Dynatech microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Percentage of dead target cells was determined as:

% dead cells =

$$\frac{\text{optical density in wells with rTNF-alpha/supernatants}}{\text{optical density in control wells}}$$

### **TNF-alpha Blocking Assay**

A mouse anti-human TNF-alpha monoclonal antibody (15C8) was used in the blocking assay (18). Cytotoxicity assays were performed as described above and anti-TNF-alpha mAb was added into the well before adding supernatants to be tested. The control was the same isotype mouse anti-ricin monoclonal antibody (2D3). The percentage of cytotoxicity inhibition was calculated.

### **RNA Studies**

RNA was prepared from  $5 \times 10^6$  cells using a single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-Chloroform extraction (19). RNA was run through Hybri-Slot<sup>TM</sup> blot on nitrocellulose membrane (BRL Cat. No. 1052MM) and hybridization was done with a kinased TNF-alpha (17) synthetic oligomer probe (sp. act.  $2 \times 10^8$  cpm/ug). The sequence used was: 5'--AAGGTCAACCTCCTCTCTGCCATCAAG--3'. Autoradiography was done using Kodak X-ray film XAR-5.

### **RESULTS**

1. **Stimulation of purified Langerhans cells releases cytotoxic activity.**

Figure 1a presents a standard curve for the MTT-colorimetric microcytotoxicity assay using recombinant TNF-alpha. The sensitivity of this assay is 0.05 units of TNF-alpha. Figure 1b is a regression curve for the linear portion of the sigmoidal dose-response. The assay was linear over the range 0.025-12 units of TNF-alpha/ml. Table 1 shows that cultures enriched for Langerhans cells stimulated with PMA/LPS release 4-5 units/ml of TNF-alpha. Cultures depleted of LCs release much less TNF-alpha (0.1U/ml). Keratinocytes stimulated in the same manner as the LCs release even less TNF-alpha (0.03 U/ml). Cell lines were used for the positive (HL-60) and negative (K562) controls for these experiments.

**2. Released cytotoxic activity is tumor necrosis factor.**

To confirm that the released cytotoxic activity was TNF-alpha, monoclonal antibody neutralization studies were performed. Figure 2a shows the representative cytotoxic response of LC enriched and LC depleted cultures both stimulated and unstimulated with appropriate positive and negative control cell lines. Figure 2b demonstrates that the cytotoxicity released from stimulated Langerhans cells is neutralized by a specific anti-human TNF-alpha murine monoclonal antibody (18).

**3. TNF-alpha mRNA is produced by stimulated Langerhans cells in vitro.**

Figure 3 shows an mRNA slot blot of cells enriched for Langerhans cells stimulated with phorbol ester and lipopolysaccharide (lane 1-3). TNF-alpha mRNA is produced by



all the cells stimulated by PMA/LPS. Lane 1 shows the quantity of TNF-alpha mRNA from the original cell population isolated from fresh skin. Lane 2 is the TNF-alpha mRNA signal obtained from the Langerhans cell enriched population. The population of cells depleted of LCs shows a weaker signal (lane 3). The negative control K562 cells also produce some TNF-alpha mRNA after being stimulated in vitro (lane 4). The positive control HL-60 cells produce abundant TNF-alpha message (lane 5). Lanes 6-10 are the corresponding unstimulated cell populations. In all cases very little message for TNF-alpha is produced.

## DISCUSSION

These studies demonstrate that purified Langerhans cells can be stimulated to produce and release TNF-alpha in vitro. The cytotoxicity of the released TNF-alpha can be neutralized by an anti-TNF-alpha antibody and LCs contain mRNA specifically hybridizing to a TNF-alpha oligomer probe. Unstimulated LCs and keratinocytes produce less than 0.1 U TNF-alpha/ml (below the detection sensitivity of the bioassay). A number of cell types including mast cells, mononuclear phagocytes and glial cells have been reported to release TNF-alpha (10). It is possible that the mRNA signal observed from the population of cells depleted of LCs is originating from keratinocytes. TNF-alpha has been recently shown to be a membrane-associated protein (20). Given the low quantity of TNF-alpha released by the LC depleted cultures it is possible that the relatively high levels of mRNA observed in the slot blot correspond to this form of the mediator. Further studies will be required to confirm this suspicion. It is also possible that the

population was incompletely depleted of LCs. However by immunoflorescent staining the depleted populations contained less than 1% LCs.

TNF-alpha is a pleiotropic mediator of the immunoinflammatory response (10). TNF-alpha is known to activate endothelial cells and granulocytes (21-22). Like IL-1, TNF-alpha promotes the growth of T cells and B cells when they have been stimulated with antigen (23-24) and may participate in antigen presentation (25). TNF-alpha (0.2-0.4 U/ml) is released during the mixed lymphocyte reaction (26), and as little as 10 U/ml augments thymidine incorporation by 60%. Thus local production of TNF-alpha by LCs has major consequences for the initiation and maintenance of the inflammatory response in the skin. Future studies will aim to determine the in situ localization of TNF in various diseases of the skin including psoriasis (27).

#### **Acknowledgements**

The expert technical assistance of Eva A. Pfendt is gratefully acknowledged.

## REFERENCES

1. Wolff, K., and Stingl, G. The Langerhans cell. *J. Invest.Dermatol.* 80,17, 1983.
2. Basset, F., Soler, P., Hance, A.J. The Langerhans' cell in human pathology. *Ann NY Acad. Sci.* 465,324, 1986.
3. Stingl, G., Katz, S., Clement, L., Green, I., Shevach, E.M. Immunologic functions of Ia-bearing epidermal Langerhans cells. *J. Immunol.* 121,2005, 1978.
4. Rowden, G., Lewis, M.G., Sullivan, A.K. Ia antigen expression on human epidermal Langerhans cells. *Nature* 268,247, 1977.
5. Klareskog, L., Malmnas-Tierlund, U.M., Forsum, U., Peterson, P.A. Epidermal Langerhans cells express Ia antigens. *Nature* 268,248, 1977.
6. Fithian, E., Kung, P., Goldstein, G., Rubenfield, M., Fenoglio, C., Edelson, R. Reactivity of Langerhans cells with hybridoma antibody. *Proc. Natl. Acad. Sci. USA* 78, 2541, 1981.
7. Groh, V., Tani, M., Harer, A., Wolff, K., Stingl, G. Leu-3/T4 expression on epidermal Langerhans cells in normal and diseased skin. *J. Invest. Dermatol.* 86,115, 1986.

8. Stingl, G., Wolff-Schreiner, E.C.H., Pichler, W.J., Gschnait, F., Knapp, W., Wolff, K. Epidermal Langerhans cells bear Fc and C3 receptors. *Nature* 258,245, 1977.
9. Braathen, L.R., Thorsby, E. Studies on human epidermal Langerhans cells: Allo-activating and antigen presenting capacity. *Scand. J. Immunol.* 11,401, 1980.
10. Nathan, C.F. Secretory products of macrophages. *J. Clin. Invest.* 79,319, 1987.
11. Larrick, J.W. and Kunkel, S.L. The role of TNF and IL-1 in the immunoinflammatory response. *Pharmaceutical Science* 5,129, 1988.
12. Sauder, D.N., Carter, C., Katz, S.I., and Oppenheim, J.J. Epidermal cell production of thymocyte activating factor (ETAF) *J. Invest. Dermatol.* 79,34, 1982.
13. Kupper, T.S., Ballard, D.W., Chua, A.O., McGuire, J.S., Flood, P.M., Horowitz, M.C. Langdon, R., Lightfoot, L. Gubler, U. Human keratinocytes contain mRNA indistinguishable from monocyte interleukin 1 alpha and beta mRNA: Keratinocyte epidermal cell-derived thymocyte factor is identical to interleukin 1. *J. Exp. Med.* 164,2095, 1986.
14. Sauder, D.N., Dinarello, C.A., Morhenn V.B. Langerhans cell production of interleukin-1. *J. Invest. Dermatol.* 12,605, 1984.

15. Liu, S-C, and Karasek, M. Isolation and growth of adult human keratinocytes in cell culture. *J. Invest. Dermatol* 71:157-162, 1978.
16. Morhenn, V.B., Wood, G.S., Engleman, E.G., Oseroff, A.R. Selective enrichment of human epidermal cell subpopulations using monoclonal antibodies. *J. Invest. Dermatol* 81 (Suppl),127s, 1983.
17. Wang, A.M., Creasey, A.A., Ladner, M.B., Lin, L.S., Strickler, J., Van Arsdell, J.N., Yamamoto, R., Mark, D.F. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 228,149, 1985.
18. Fendly, B.M., Toy, K.J., Creasey, A.A., Vitt, C.R., Larrick, J.W., Yamamoto, R., and Lin, L.S. Murine monoclonal antibodies defining neutralizing epitopes on tumor necrosis factor. *Hybridoma* 6,359, 1987.
19. Chomczynski, P. and Sacchi, N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Anal. Biochem.* 162,156, 1987.
20. Kriegler, M., Perez, C., Defay, K., Albert, I., Lu, S-D. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* 53,45, 1988.

21. Nawroth, D.P. and Stern, D.M. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.* 163,740, 1986.
22. Larrick, J. W., Graham, D., Toy, K., Lin, L.S., Senyk, G. Fendly, B.M. Recombinant tumor necrosis factor causes activation of human granulocytes. *Blood* 69,640, 1987.
23. Kehrl, J.H., Miller, A. and Fauci, A. S. Effect of tumor necrosis factor alpha on mitogen activated human B cells. *J. Exp. Med.* 166:768-672, 1987.
24. Lee, J.C., Truneh, A. Smith, M.F. Jr., Tsang, K.Y. Induction of interleukin 2 receptors (TAC) by tumor necrosis factor in YT cells. *J. Immunol.* 139,1935, 1987.
25. Gottlieb, A.B., Lifshitz, B., Fu, S.M., Staino-Coico, L., Wang, C.Y., Carter, D.M. Expression of HLA-DR molecules by keratinocytes and presence of Langerhans cells in the dermal infiltrate of active psoriatic plaques. *J. Exp. Med.* 164,1013, 1986.
26. Shalaby, M.R., Espevik, T., Rice, G.C., Ammann, A. J., Figari, I.S., Ranges, G.E., Palladino, M.A. Jr. The involvement of human tumor necrosis factors-alpha and beta in the mixed lymphocyte reaction. *J. Immunol.* 141,499, 1988.

27. Oxholm A, Oxholm P., Staberg B. Reduced density of T6-positive epidermal Langerhans' cells in uninvolved skin of patients with psoriasis. Acta. Derm. Venereol. 67,8, 1987.

**Table 1: Induction of TNF-alpha from Langerhans**

**Enriched Populations of Cells**

Cell population	Stimulation (PMA/LPS)	
	+	-
Langerhans enriched	4.53 $\pm$ 1.78 <sup>a,b</sup>	$\leq 0.04$
Langerhans depleted	0.10 $\pm$ 0.04	$\leq 0.04$
Keratinocytes	0.03 $\pm$ 0.01	$\leq 0.04$
Positive control (HL60)	2.65 $\pm$ 0.9	$\leq 0.04$
Negative control (K562)	$\leq 0.04$	$\leq 0.04$

<sup>a</sup> N=5 experiments

<sup>b</sup> TNF-alpha Units/ml; mean  $\pm$  standard deviation



**Figure 1:**

- a) L929 MTT cytotoxicity assay: rTNF-alpha standard curve.
- b) Linear regression for the standard curve.

**Figure 2:**

- a) **Stimulated Langerhans cells release cytotoxic activity.**

L929 assay demonstrating cytotoxic activity released from LPS and PMA stimulated or unstimulated control Langerhans cell populations. Populations enriched (+) or depleted (-) of Langerhans cell are shown. Cytotoxic activity of stimulated positive control HL60 and stimulated negative control K562 cells are also shown. Cytotoxicity above 20% is significant (see standard curve Figure 1). Representative of three experiments is shown.

- b) **Cytotoxic activity is neutralized by specific anti-TNF-alpha monoclonal antibody.**

L929 assay demonstrating cytotoxic activity of LPS/PMA stimulated Langerhans enriched (+) and depleted (-) populations of cells is neutralized by specific anti-human TNF-alpha monoclonal antibody (15C8 at 40 ug/ml).

**Figure 3:**

**Slot Blot Analysis of TNF-alpha mRNA Expression from Langerhans Cells.**

RNA was prepared from  $5 \times 10^6$  cells, slot blotted and probed with an end-labeled TNF-alpha specific oligomer probe as described in the methods.

**Unseparated skin cell population-**

Slot 1: LPS/PMA;                      slot 6:                      no induction

**Langerhans enriched population-**

Slot 2: LPS/PMA;                      slot 7:                      no induction

**Langerhans depleted population-**

Slot 3: LPS/PMA;                      slot 8:                      no induction

**Negative control K562 cells-**

Slot 4: LPS/PMA;                      slot 9:                      no induction

**Positive control HL60 cells-**

Slot 5: LPS/PMA;                      slot 10:                      no induction









